THE SPECIFIC CAPSULAR POLYSACCHARIDE OF *Pneumococcus* TYPE IV

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ABSTRACT

The specific, capsular polysaccharide (SIV) of *Pneumococcus* Type IV is shown to contain acetal-linked pyruvic acid, D-galactose, 2-amino-2-deoxy-D-galactose, 2-amino-2-deoxymannose, and 2-amino-2,6-dideoxy-D(?)-galactose in the molar ratios 3:3:3:2:3, the amino sugars presumably being *N*-acetylated.

INTRODUCTION

The immunologically specific, capsular polysaccharide (SIV) of *Pneumococcus* Type IV has thus far escaped detailed examination, in part because of a discouraging initial report[1]. Since then, improved methods of isolation[2], and the discovery that SIV contained pyruvic acid[3] and developed group-specificity[4] on depyruvylation, stimulated renewed interest in the substance and led to the present studies of the purification of SIV and its chemical composition.

MATERIALS AND METHODS

SIV was prepared by Dr. Paul A. Rebers in 1958 at the Institute of Microbiology, Rutgers, the State University, New Brunswick, N. J., much as previously described[2], and was isolated as the lithium salt. The Type IV pneumococci were grown at Fort Detrick, Md., courtesy of Dr. Riley D. Housewright, and were killed with phenol before collection.

Antipneumococcal (anti-Pn) horse sera were obtained from the Laboratories of the New York City and New York State Departments of Health through Drs. Paul S. May and Kenneth Amiraian. Analyses for antibody nitrogen precipitated in homologous and cross-reactions in anti-Pn sera were carried out as described in earlier papers[5,6].

Reference sugars were commercial preparations, recrystallised when necessary to give products that were pure by g.l.c. Evaporations were performed at 25–30° under

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reduced pressure in a rotary evaporator. Spectrophotometric absorbances were measured in 10-mm semimicro cuvettes.

SIV (0.2–1.0 mg) was hydrolysed by 3M hydrochloric acid for 8 h at 100° under nitrogen. After filtration through sintered glass, partial removal of hydrochloric acid from hydrolysates was effected by rotary evaporation. Neutral and amino sugars were separated by elution from columns (6 × 0.6 cm) of AG-50W x8(H^+) resin (Bio-Rad Laboratories) with water and 2M hydrochloric acid, respectively[4].

Galactose, the only neutral sugar present, was determined (*a*) in intact SIV and (*b*) after hydrolysis and separation from amino sugars by ion-exchange, by the following methods: cysteine–H_2SO_4[7], phenol–H_2SO_4[8], and modified cysteine–H_2SO_4[9], as well as by "Galactostat" (D-galactose oxidase, Worthington Biochemical Corp, Freehold, N. J., U. S. A.) on a decreased scale, with omission of glycine buffer.

Amino sugars in eluates were determined by the Gatt–Berman modification[10] of the Elson–Morgan assay. *N*-Acetylamino sugars liberated on hydrolysis with dilute acid were determined by a modification[11] of the Morgan–Elson assay.

For g.l.c., solutions of neutral and amino sugars were separately evaporated in 5-ml pear-shaped flasks. The last traces of water and hydrochloric acid were removed by co-distillation with ethanol–benzene (4:1). After addition of mannitol (40 μg) as internal standard, conversion into *O*-trimethylsilyl (TMS) derivatives was performed as described by Sweeley *et al.*[12], except that incubation was for 30 min at 37°. Excess solvents were removed by rotary evaporation, and the dry residue was reconstituted in hexane (25–100 μl). Aliquots (1–2 μl) were chromatographed on a column (6 ft. × 0.4 cm) of coiled glass packed with 3% S.E.30 on Supelcoporte (Supelco Inc., Bellaforte, Pa.) at 185° in a Packard modular gas chromatograph equipped with a dual flame-ionisation detector.

Pyruvic acid was estimated by titration, and by the method of Sloneker and Orentas[13] on a decreased scale. Phosphorylcholine was determined according to the method of Gourley *et al.*[14], as a test for contamination by C-substance.

Phosphorus was determined in ashed samples by a micro-modification of the method of Chen *et al.*[15].

RESULTS AND DISCUSSION

A small proportion of C-polysaccharide in SIV could not be removed by fractionation on Sephadex G-25 and G-50. SIV was not eluted from DEAE-Sephadex A25 (in the acetate form) with sodium acetate buffer (pH 6.5) alone, but this washed out most of the C-substance. SIV was then eluted as a sharp peak with 0.5M sodium chloride in buffer and was followed much later by a second small peak (see below). The recovered SIV (71%) was almost free of C-substance, and its specific activity and content of monosaccharides were identical with those of unfractionated SIV. Immunoelectrophoresis in agarose at pH 8.6 (barbital buffer) gave no evidence of heterogeneity.

The much smaller, second peak comprised ~2% of the applied polysaccharide. It contained glucose, galactose, pyruvic acid, and 2-amino-2-deoxy-D-glucose (glucos-

amine) in approximately equimolar amounts, with no other amino sugars. This substance precipitated 90% as much rabbit anti-Pn IV as did SIV, and only 3.5% on depyruvylation, which is 83% of the amount precipitated by depyruvylated SIV. It also showed some cross-reactivity with antibodies to pneumococcal C substance. The origin of this variant of SIV is unknown.

Hydrolysis of SIV by acid

Hydrolysis by 2M hydrochloric acid for 5 h at 100° gave galactose as the only neutral sugar. By g.l.c., several peaks appeared in the amino sugar fraction, all but two being identified by co-chromatography with standards as 2-amino-2-deoxygalactose (galactosamine) and 2-amino-2-deoxymannose (mannosamine). A major peak (R_{GalN} 0.43) and a minor peak (R_{GalN} 0.25) were identified later as due to 2-amino-2,6-dideoxygalactose (fucosamine).

Hydrolysis of 200–1000 μg of SIV with hydrochloric acid, for 5 min to 8 h at 100° under nitrogen, showed that 3M acid for 8 h, the longest period used, gave maximal liberation of galactosamine and mannosamine, 18.5 and 12.5% of the weight of SIV, respectively. Under these conditions, standard mixtures of hexosamines were recovered in essentially 100% yield. Hydrolysis of SIV with 4M hydrochloric acid for 3–4 h gave only 16 and 9.5% of the two aminohexoses.

Assay of SIV, without prior hydrolysis, by the three spectrophotometric assays gave 17.5–20% of galactose. Analysis by g.l.c., after hydrolysis with 3M hydrochloric acid (for 8 h at 100° under nitrogen) and separation from amino sugars by ion-exchange, gave only 13.1% of galactose, indicating considerable destruction. Galactose alone, heated in the same way, gave 92% recovery. Manipulative losses of control galactose, galactosamine, and mannosamine solutions on ion-exchange columns were <1%. Galactose released from SIV by hydrolysis with 3M hydrochloric acid for 2 h at 100° was separated from amino sugars as described. Acid was removed by passage through Amberlite IR-45 (previously washed with M hydrochloric acid and water to remove contaminants). Analysis of the neutralised eluate by g.l.c. and cysteine–H_2SO_4 gave values equivalent to those with "Galactostat", demonstrating that galactose in SIV was exclusively the D enantiomer.

Pyruvic acid was first reported as a component of bacterial polysaccharides by Sloneker and Orentas[13], and since then has been found in many other bacterial polysaccharides[16]. Treatment of SIV with M hydrochloric acid for 30 min at 100° liberated ~9% of pyruvic acid, and even 10mM hydrochloric acid gave almost complete release of the acid after 10 min (Fig. 1, ref. 4). After depyruvylation with 10mM hydrochloric acid for 30 min at 100°, SIV (3 mg) was fractionated on a column (93 × 1 cm) of Sephadex G-25 previously calibrated with SIV and galactose. It showed a single peak in the SIV region and yielded 8.8% of pyruvic acid, with no monosaccharides, in the region of small molecular weight. Analysis of the recovered, depyruvylated polysaccharide (dp-SIV), after treatment with M hydrochloric acid for 30 min at 100°, showed 0.6% of pyruvic acid. The composition of dp-SIV was otherwise the same as that of SIV. By contrast, hydrolysis with 40mM hydrochloric

acid, for periods up to 8 h at 100°, liberated not only pyruvic acid from SIV but also *N*-acetylhexosamines, as did hydrolysis for more than 100 min with 10mM hydrochloric acid at 100°. Fractionation on Sephadex G-50 (fine; column 107 × 0.95 cm) of depyruvylated (10mM HCl, 100°, 3 and 7 min) SIV (5 mg) showed identical sharp peaks having the same elution volume as SIV, indicating that negligible depolymerisation had occurred. A sample depyruvylated for 30 min showed a small "tail" indicating slight degradation.

Treatment of SIV with 0.1M sodium hydroxide for 1 h at room temperature, followed by dialysis, resulted in the passage of 0.6% of pyruvic acid into the dialysate. Treatment of the non-dialysable portion with M sodium hydroxide for 1.5 h at 0° liberated a further 1.1% of pyruvic acid. The recovered, non-dialysable material contained 7.8% of pyruvic acid and had an unchanged content of monosaccharides, with no change in precipitating capacity *versus* rabbit anti-Pn IV.

The ease of liberation of pyruvic acid from SIV by dilute acid and the stability to alkali indicate an acetal linkage, probably to galactose, as this sugar is oxidized by periodate only after SIV is depyruvylated. Acetal-linked pyruvic acid should have titratable COOH groups; accordingly, SIV (5 mg) in deionised water (2.5 ml) was applied to AG-50W x8(H^+) resin (7 ml) and eluted to 25.0 ml with deionised water. The resin had been previously equilibrated with deionised water and a 25.0-ml blank collected. The solution was titrated (pH meter) with 10mM sodium hydroxide under nitrogen. A blank was similarly titrated. End-points were taken as the points of inflexion on the titration curves; 0.62 and 0.06 ml of alkali, respectively. The normality of SIV was 2.24×10^{-4} N; calculated pyruvic acid, 9.9%. Therefore, the COOH groups of pyruvic acid in SIV are not esterified, confirming the acetal nature of the linkage.

The unusual change from type-specificity to group-specificity, arising from the cleavage of pyruvic acid from SIV, has been described[4].

The SIV used contained 0.95% of total P; this was due mainly to C-polysaccharide; a C-free preparation showed 0.25% of P.

The ash content of SIV was determined by combustion with sulfuric and nitric acids. A flame test showed predominantly lithium, lithium chloride having been used as electrolyte in the preparation of SIV. Equimolar amounts of lithium and pyruvic acid were found. The data on the composition of SIV and DEAE-Sephadex-purified SIV are summarised in Table I.

In the cysteine–H_2SO_4 reaction, SIV gave maximal absorption at 400 nm, indicating a shift from the galactose maximum of 412 nm due to the presence of a methylpentose or 6-deoxyhexose (fucose maximum, 398 nm), possibly resulting from the deamination of the unidentified amino sugar in the assay. Indeed, passage of the hydrolysate of SIV through AG-50W x8(H^+) and analysis of the aqueous eluate gave maximal absorption at 412 nm, typical of galactose.

SV contains, *inter alia*, L-fucosamine (2-amino-2,6-dideoxy-L-galactose) and pneumosamine (2-amino-2,6-dideoxy-L-talose)[17], while one of the sugars of SXII is also L-fucosamine[18]. SIV (5 mg) was hydrolysed (4M HCl, 100°, 3 h) and fractionated

TABLE I

COMPONENTS OF Pn SIV[a] AND DEAE-SEPHADEX-PURIFIED SIV (SIV')

Component (%)[b]	*SIV*	*SIV'*	*Molar Ratios*
"Anhydro-D-Galactose"	18	17.5	3
"Anhydro-D-Galactosamine"	16.5	17.5	3
"Anhydromannosamine"	11	11	2
"Anhydro-D(?)-fucosamine"	15.5	16.5	3
("Anhydroglucose")	<1		
("Anhydroglucosamine")	<2		
Phosphorylcholine	0.6	0.1	
Pyruvyl [$H_3C(CO)CO_2H$]	8.5	8	3
N-acetyl (calculated)	11.5	12	3
Phosphorus (as PO_4)	3	0.8	
Ash (as lithium)	0.7		3

[a]As prepared by Dr. P. A. Rebers. [b]To nearest 0.5% unless under 1%.

on AG-50W x8(H^+) resin (column, 42 × 0.8 cm) by elution with 0.33M hydrochloric acid at 2 ml/h. It gave a peak (retention volume R_{ManN} 1.62) which corresponded with fucosamine from SV chromatographed on the same column (R_{ManN} 1.65). No peak corresponding to pneumosamine (R_{ManN} 2.15) was observed. Crumpton[19] reported R_{ManN} 1.64 and 2.07 for fucosamine and pneumosamine. Analysis by g.l.c. at 160° of fucosamine, isolated from SIV as above, gave one peak at a retention time of 5.25 min which co-chromatographed exactly with fucosamine similarly isolated from SV and SXII. With a temperature programme of 150° for 1 min, 7°/min up to 200°, fucosamine from SIV and SV was eluted after 4.75 min (179°), whereas pneumosamine was eluted as 3 peaks at 4.6 (177°), 5.0 (181°), and 5.2 min (183°), with relative heights of 1:2.4:1.5. With L-fucosamine from SXII as standard, SIV was found to contain 14.5% of fucosamine after hydrolysis (3M HCl, 100°, 8 h).

Edstrom[20] reported that formation of a cyanine dye–polyanion complex resulted in a shift of wavelength characteristic of the number of anionic sites per monosaccharide residue. SIV (2.9 μg) gave a sharp peak (O.D. 0.560) with a wavelength maximum at 653 nm, suggestive of one anionic site (pyruvic acid) per 4–5 monosaccharide residues and consistent with the presence of one pyruvic acid group per residue of galactose. Depyruvylation of SIV for 3, 7, and 30 min abolished the maximum at 653 nm, the products giving spectra identical to that of the dye itself with a maximum at 508 nm.

Recovery of SIV from the specific precipitate with anti-Pn IV

SIV was dissociated from the specific precipitate formed by approximately equivalent amounts of SIV and partially purified anti-Pn IV. The globulin in the washed precipitate was denatured in the cold with 5% trichloroacetic acid[21], and the denatured protein was washed once with the same solvent. The solution was boiled to decompose the acid and evaporated to dryness. Analysis showed the same monosaccharide constituents as for the original SIV, and a pyruvic acid content of 8.4%.

Isolation and characterisation of the component sugars of SIV

SIV (414 mg) was depyruvylated with 10mM hydrochloric acid (200 ml) for 15 min at 100° under nitrogen, and recovered after exhaustive dialysis. The depyruvylated SIV (*ca.* 377 mg) was then hydrolysed with 3M hydrochloric acid (267 ml, 100°, 7.5 h). The concentrated hydrolysate was freed as much as possible from hydrochloric acid by evaporation with ethanol–benzene (4:1), before application to a column (62 × 2.6 cm) of AG-50W x8(H^+) resin and elution first with water (360 ml). Fractions of 20 ml were collected at 15 ml/h. Elution was continued with M hydrochloric acid (1000 ml) as above. The resulting fractionation is shown in Fig. 1.

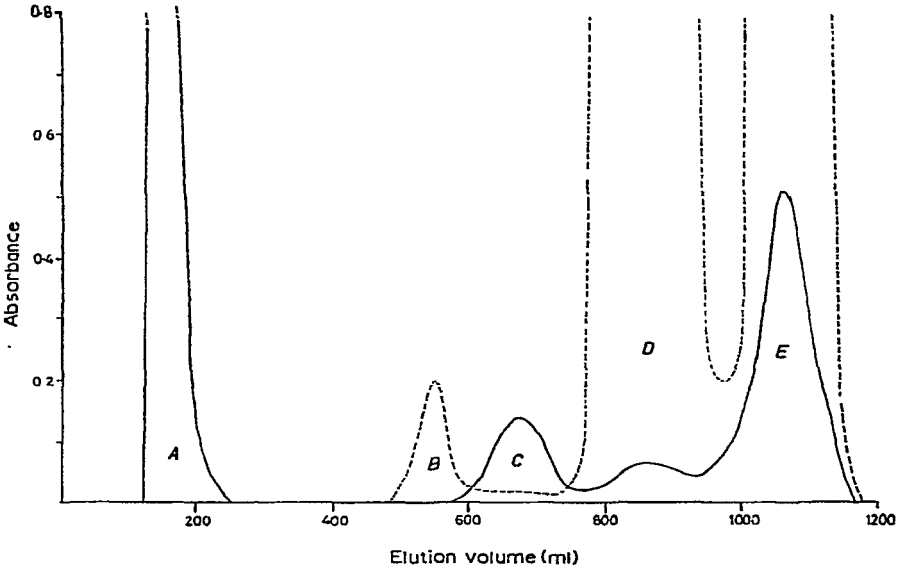

Fig. 1. Fractionation of depyruvylated SIV on AG-50W x8(H^+) resin after hydrolysis (3M HCl, 7.5 h, 100°), equilibrated at 4°. Elution was first with water (360 ml), then with M HCl. Aliquots of the 20-ml fractions were analysed for galactose (——), and, without further hydrolysis, amino sugar (– – –).

Peak *A* (fractions 6–12, Fig. 1) yielded crystals which were recrystallised 3 times from moist ethanol. The dried substance (1.2 mg) was dissolved in water (6.0 ml), and aliquots (0.2 ml) were analysed by g.l.c. Three peaks characteristic of galactose were shown, with areas equivalent to those of an equal amount of authentic galactose. Co-chromatography of sample and standard gave completely superimposed peaks. The galactose of SIV and standard D-galactose gave equivalent values in both the cysteine–H_2SO_4 and "Galactostat" assays, showing that the sugar in SIV is the D isomer. Its melting point, after exhaustive drying *in vacuo*, was 166–167°; mixed melting-point with authentic D-galactose, 166–167°.

Peaks *B* and *C* (Fig. 1) were oligosaccharides and will be described in another publication.

Peak *D* (fractions 38–47, Fig. 1) contained (g.l.c.) only glucosamine and mannosamine hydrochlorides. The mixture was dried, dissolved in 0.33M hydrochloric acid (5 ml), and fractionated on a column (81 × 2.6 cm), of AG-50W x8(H^+) resin previously equilibrated with 0.33M acid at 4°. Elution with 0.33M hydrochloric acid was initially at 2 ml/h in 5.1-ml fractions up to 1000 ml, then at 4 ml/h in 12-ml fractions. Aliquots (0.5 ml) were analysed for hexosamines (Fig. 2). Aliquots from the maximum of each peak were analysed by g.l.c. Peak *D1* contained glucosamine, peak *D2* mannosamine, and peak *D3* galactosamine. Peak *D2* had R_{GlcN} 1.08 and *D3*, 1.18; Crumpton[19] reported 1.07 and 1.20 for mannosamine and galactosamine.

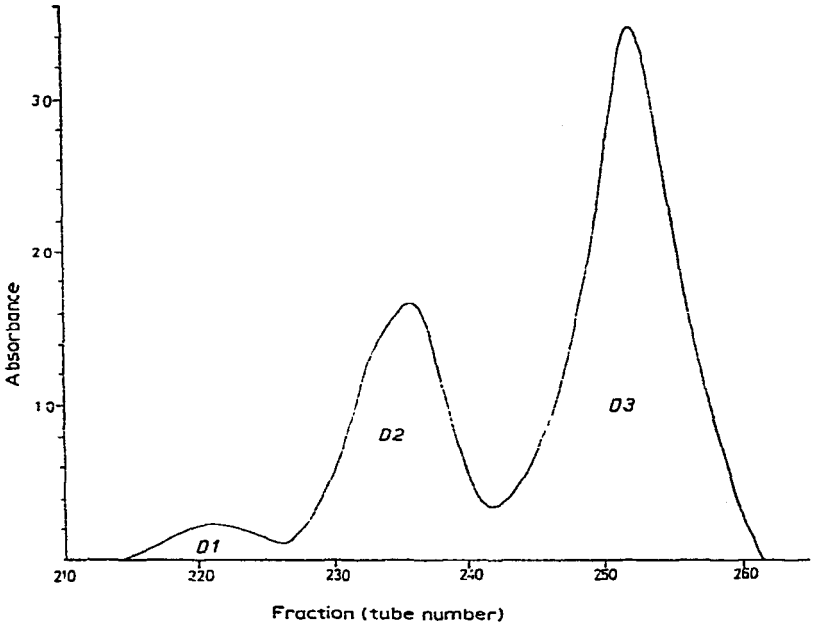

Fig. 2. Fractionation of peak *D* (Fig. 1) on AG-50W x8(H^+) resin. Elution with 0.33M HCl was initially at 2 ml/h (5.1-ml fractions) for 1000 ml, thereafter at 4 ml/h (12-ml fractions). Aliquots were analysed for total hexosamines (——).

Comparison of the areas of peaks *D1* and *D2*, with the assumption that the area of peak *D2* corresponds to 11% "anhydromannosamine", yields an estimate of <2% "anhydroglucosamine" for SIV. This may have originated from the associated glucosamine-containing polysaccharide obtained from DEAE-Sephadex-purified SIV. Fraction *D3* was concentrated and the galactosamine obtained as its crystalline hydrochloride. Analysis of the twice-recrystallised substance obtained from SIV, and of authentic D-galactosamine hydrochloride, by "Galactostat" showed that the D isomer was exclusively present in SIV. Fractions *D1* and *D2* did not react with "Galactostat". The galactosamine hydrochloride from SIV melted at 180° (lit. 178–180°), unchanged by admixture with authentic D-galactosamine hydrochloride.

The crystals gave a single, sharp peak at 199° on g.l.c., which co-chromatographed with standard galactosamine.

Peak *E* (Fig. 1). Analysis by g.l.c. of tubes 49–58 showed only fucosamine. Concentration yielded needle-like crystals which were recrystallised 4 times from moist acetone; melting point 175–178° (dec.) with charring commencing at 155°; D-fucosamine hydrochloride m.p. 177–183° (dec.)[22] and 170–175° (dec.)[23]; L-fucosamine hydrochloride[24] m.p. 192–193°. Therefore, fucosamine in SIV is probably the D isomer.

Analysis of an aqueous solution (217 μg/ml) of the fucosamine of SIV by the Gatt–Berman modification[10] of the Elson–Morgan reaction gave an absorption maximum initially at 545 nm and declining to 530 nm over 21.5 h.

In the cysteine–H_2SO_4 assay, the fucosamine of SIV gave an absorption maximum (400 nm) identical to that of fucose and equivalent to 25% of that of an equimolar amount of fucose. Cysteine–H_2SO_4 analysis of SIV, together with standards of fucose and galactose, at both 400 and 420 nm, enables an apparent fucose content of SIV to be calculated by solution of the two simultaneous equations. A value of 4.2% was obtained, equivalent to 17% of fucosamine in SIV, as calculated above. This agrees well with an average of 14.5% obtained by g.l.c. after hydrolysis with acid. SIV is thus the third pneumococcal polysaccharide found to contain a fucosamine, the others being SV[17] and SXII[18]. Similarly, only SIX[25] and SXIX[26] have been previously shown to contain mannosamine.

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REFERENCES

1 M. HEIDELBERGER AND F. E. KENDALL, *J. Exp. Med.*, 53 (1931) 625.
2 M. HEIDELBERGER, C. M. MACLEOD, H. MARKOWITZ, AND A. S. ROE, *J. Exp. Med.*, 91 (1950) 341.
3 M. HEIDELBERGER, W. F. DUDMAN, AND W. NIMMICH, *J. Immunol.*, 104 (1970) 1321.
4 J. D. HIGGINBOTHAM, M. HEIDELBERGER, AND E. C. GOTSCHLICH, *Proc. Nat. Acad. Sci. (U. S.)*, 67 (1970) 138.
5 M. HEIDELBERGER AND P. A. REBERS, *J. Amer. Chem. Soc.*, 80 (1958) 116.
6 M. HEIDELBERGER AND J. M. TYLER, *J. Exp. Med.*, 120 (1964) 711.
7 Z. DISCHE, *J. Biol. Chem.*, 167 (1947) 189.
8 M. DUBOIS, K. A. GILLES, J. K. HAMILTON, P. A. REBERS, AND F. SMITH, *Anal. Chem.*, 28 (1956) 350.
9 Z. DISCHE AND A. DANILCHENKO, *Anal. Biochem.*, 21 (1967) 119.
10 R. GATT AND E. R. BERMAN, *Anal. Biochem.*, 15 (1966) 167.
11 J. L. REISSIG, J. L. STROMINGER, AND L. F. LELOIR, *J. Biol. Chem.*, 217 (1955) 959.
12 C. C. SWEELEY, R. BENTLEY, M. MAKITA, AND W. W. WELLS, *J. Amer. Chem. Soc.*, 85 (1963) 2497.
13 J. H. SLONEKER AND D. G. ORENTAS, *Nature (London)*, 194 (1962) 478.
14 W. K. GOURLEY, C. D. HAAS, AND S. BAKERMAN, *Anal. Biochem.*, 19 (1967) 197.
15 P. S. CHEN, T. Y. TORIBARA, AND H. WARNER, *Anal. Chem.*, 28 (1956) 1786.
16 C. J. LAWSON, C. W. MCCLEARY, H. I. NAKADA, D. A. REES, I. W. SUTHERLAND, AND J. E. WILKINSON, *Biochem. J.*, 115 (1969) 947.
17 S. A. BARKER, J. S. BRIMACOMBE, M. J. HOW, M. STACEY, AND J. M. WILLIAMS, *Nature (London)*, 189 (1961) 303; J. S. BRIMACOMBE AND M. J. HOW, *J. Chem. Soc.*, (1962) 5037; (1963) 3886.

18 J. A. CIFONELLI, P. A. REBERS, M. B. PERRY, AND J. K. N. JONES, *Biochemistry*, 5 (1966) 3066.
19 M. J. CRUMPTON, *Biochem. J.*, 72 (1959) 479.
20 R. D. EDSTROM, *Anal. Biochem.*, 29 (1969) 421.
21 M. HEIDELBERGER, Z. DISCHE, W. B. NEELY, AND M. L. WOLFROM, *J. Amer. Chem. Soc.*, 77 (1955) 3511.
22 N. SHARON, I. SHIF, AND U. ZEHAVI, *Biochem. J.*, 93 (1964) 210.
23 M. J. CRUMPTON AND D. A. L. DAVIES, *Biochem. J.*, 70 (1958) 729.
24 R. KUHN, W. BISTER, AND W. DAFELDECKER, *Ann.*, 628 (1959) 186.
25 J. D. HIGGINBOTHAM, A. DAS, AND M. HEIDELBERGER, *Biochem. J.*, 126 (1972) 225; A. DAS, J. D. HIGGINBOTHAM, AND M. HEIDELBERGER, *Biochem. J.*, 126 (1972) 233.
26 T. MIYAZAKI AND T. YADOMAE, *Carbohyd. Res.*, 16 (1971) 153.